SISTER CHROMATID EXCHANGES IN TRITIUM-LABELED CHROMOSOMES[1]

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By allowing chromosomes to duplicate once in the presence of tritium-labeled thymidine and then following the distribution of the labeled DNA (deoxyribonucleic acid) in subsequent divisions in the absence of labeled precursors, TAYLOR, WOODS and HUGHES (1957) demonstrated that the original DNA is conserved during duplication and distributed in a very precise manner at each cell division. In addition the chromosomes of the broad bean (*Vicia faba*) were shown to be duplexes, i.e., they are composed of two strands before duplication. At the first mitosis after labeling occurs in interphase, all of the daughter chromosomes are labeled. However, the labeled chromosomes do not distribute the labeled DNA equally to their descendants. At the next division after a duplication in the absence of labeled precursors, they regularly produce one labeled daughter chromosome and one unlabeled daughter chromosome. These facts can only be explained if one original, unlabeled strand and one new, labeled strand were received by each of the original labeled chromosomes. At the next division the labeled strand and a newly replicated unlabeled strand are passed on to one daughter chromosome. The unlabeled strand and another newly replicated, unlabeled strand are received by the other daughter. When these second division chromosomes were observed in colchicine-blocked metaphases in which the daughter chromosomes (referred to as sister chromatids before anaphase separation in mitosis) lie somewhat separated except at the centromeres, reciprocal exchange of segments, sister chromatid exchanges, could be observed. For example, a chromatid might be labeled along only part of its length, but in every such instance the other chromatid was labeled in the segment lying opposite the unlabeled segment.

The experiments described below were designed to test the chromosomes of a liliaceous plant for the two-strand property. When the existence of the chromosomal duplex was confirmed and the sister chromatid exchanges proved to be frequent and amenable to analysis, additional material was prepared for a quantitative study of the types and frequency of exchanges. The analysis so far has given relatively little information on the natural frequency of sister chromatid exchanges, but a discovery concerning the structural relationship of the two strands of the duplex has emerged, which is of considerable intrinsic interest in addition to its importance for further studies on chromatid exchanges and crossing over.

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MATERIALS AND METHODS

Roots on bulbs of *Bellevalia romana* were grown in mineral solutions containing 2–3 μg of thymidine-H^3 (specific activity 300–400 $\mu c/\mu$ mole) per ml of solution. The thymidine-H^3 was part of the material prepared by DR. W. L. HUGHES for our original experiments. The specific activity is lower than originally estimated because of the presence of thymidine derivatives produced in the exchange reaction. These derivatives were not detected on the paper chromatograms first used for the separation of the labeled thymidine because they do not absorb ultraviolet light at the same wave lengths as thymidine. However, they have the same or nearly the same Rf value as thymidine in the solvents used. The derivatives were discovered when carrier was added and the thymidine recrystallized (HUGHES, personal communication). In collaboration with DR. W. L. HUGHES of the Brookhaven National Laboratory, Schwarz Laboratories, Inc., Mount Vernon, New York, have prepared and purified thymidine-H^3 of high specific activity, which is now available commercially. Samples of this material have been available for recent experiments. Since the mixture as well as the purified product labels only the chromosomes, the impurities did not seriously interfere in these experiments.

Two procedures are used for labeling the chromosomes and following the distribution of the label in subsequent divisions. All roots were grown at 25±.5°C. After 6–10 hours in the isotope solution roots were either fixed or washed and transferred to isotope free solutions. In one series of experiments designed to observe the division of labeled chromosomes in the absence of colchicine, roots were fixed immediately after removal from the isotope solution and after ten and 18 hours in the solutions free of the isotope. Since labeled anaphases appeared infrequently in these preparations because of the unsynchronized division cycle of the cells, subsequent fixations were made after roots had been in isotope solution six hours, isotope free solution 12 hours and in colchicine for 12–14 hours. The latter preparations yield diploid, colchicine-blocked metaphases (c-metaphases) at the second division after labeling. The chromosomes in these cells are easier to spread and analyze than in the tetraploid cells. However, when analysis of sister chromatid exchanges was made, it became necessary to have all of the chromosomes derived from the original labeled complement in one cell. Therefore, colchicine was used as in the original experiments (TAYLOR *et al.* 1957). In this series of experiments roots were placed in the isotope solution for 6–8 hours and then fixed after ten hours in the colchicine solution. After some preliminary trials colchicine was used at a concentration of 600μg per ml of mineral solution. This concentration is almost completely effective in blocking anaphase and cell division (cytokinesis), but does not prevent reorganization of an interphase nucleus and a second duplication of the chromosomes.

Cells were fixed in acetic-alcohol (3:1), hydrolyzed and stained by the Feulgen procedure. Very much flattened squash preparations were made. The preparations were frozen on dry ice and autoradiographs prepared as described previously (TAYLOR *et al.* 1957; TAYLOR 1956).

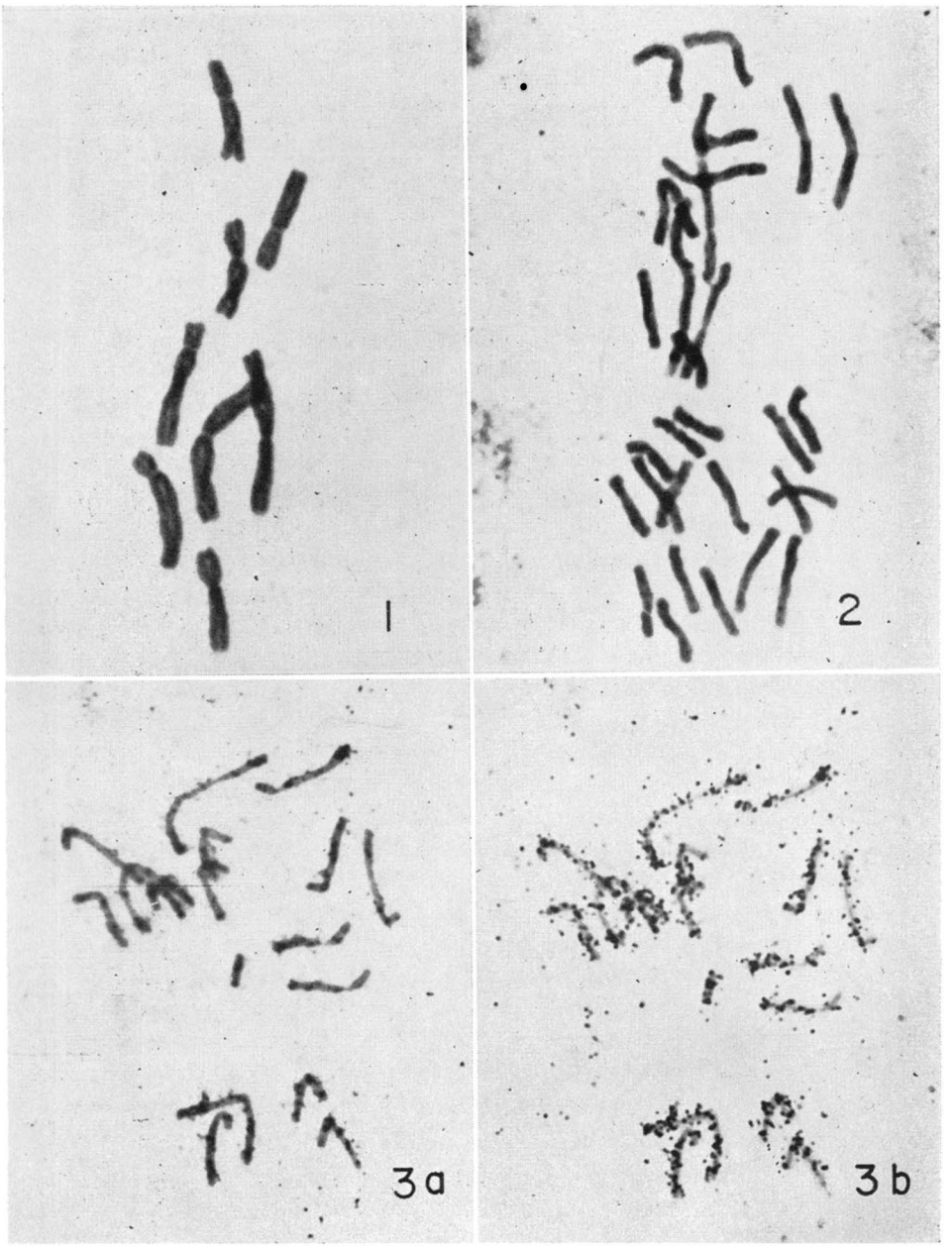

FIGURE 1.—C-metaphase chromosomes of Bellevalia (diploid). × 1650.

FIGURE 2.—Tetraploid c-anaphase: the sister chromatids have separated completely. × 1500. × 1500.

FIGURE 3.—Flattened anaphase at the first division following labeling with tritium-thymidine. × 1150. 3a.—Focal level of chromosomes. 3b.—Focal level of grains.

Bellevalia is advantageous for these experiments because it has only eight large chromosomes in diploid cells (Figure 1). Three morphological types are readily distinguished. The largest chromosome, designated chromosome I, has a median centromere. The medium sized one, chromosome II, has arms of very unequal length and is easily distinguished from chromosomes III and IV. Unfortunately, the smallest chromosomes, III and IV, are so nearly alike morphologically that they cannot be distinguished from each other. Therefore, they will be treated as one class in the analyses. The sister chromatids lie together like those in Figure 1 during most of the c-metaphase. However, for a short period in late c-metaphase, they separate as shown in Figure 4a. Finally, the sister chromatids move apart and sisters cannot regularly be recognized by their position (Figure 2).

EXPERIMENTAL RESULTS

After ten hours in the isotope solution many interphase nuclei, and nearly all of the prophase, metaphase and anaphase chromosomes were uniformly labeled from end to end in so far as autoradiographs allow determination of this characteristic (Figure 3). Certainly all parts of every chromosome in the flattened anaphase figures were labeled. Although unlabeled sectors might escape notice in some cases with grain densities as low as those shown in the photograph (Figure 3), many of the figures had two to three times the grain density shown. These were unsatisfactory for grain counting, but they showed that unlabeled sectors do not exist at the first division following incorporation of the isotope. At least they do not under the conditions of these experiments, in which the isotope was present during the whole period of duplication of the chromosomes observed. The c-metaphases in preparations fixed after 8 hours in the isotope and 10 hours in colchicine also showed all chromatids labeled throughout their length.

By counting grains over pairs of daughter chromosomes similar to those shown in Figure 3b, data on the equality of the distribution of isotope was obtained. The data are recorded in two ways in Figure 7. First the number of grains over a chromosome in a right hand anaphase group was plotted along the abscissa, and then the number of grains over its sister in the other anaphase group was plotted along the ordinate. The points at the intersection of coordinates plotted in this way, fall on a line with a slope of one when the counts are equal. The experimentally determined points fall near the line with no significant deviations. The two counts showing the greatest deviation when tested by chi square for a 1:1 ratio have a P value between .30–.40.

Another way of plotting the data is shown in the right hand corner of the same graph (Figure 7). The ratio of isotope in each two daughter chromosomes is obtained by dividing the number of grains over the one with the higher number by the number of grains over the one with the lower number. Then the deviations of these ratios from unity are plotted as a frequency distribution. The frequency polygon appears to fit a normal curve, which indicates that the variations are random, and therefore, the distribution of label may be considered equal.

However, a more convincing test, for the precision of the segregation of the original and the newly formed DNA, can be made at the second division. Fortunately, the pool of labeled precursors is small and is depleted in a very short time after the roots are removed from the labeled solutions. To obtain cells with chromosomes that had replicated once after labeling, roots were allowed to grow for 6–8 hours in the isotope solution, then 10 hours in mineral solution free of isotope and finally 12–14 hours in colchicine solution to accumulate a number of c-metaphases. Some of these diploid c-metaphases contained labeled chromosomes. The chromosomes had one chromatid labeled and one free of label at any level along the chromosome (Figure 4). However, one of the striking features was the high frequency of sister-chromatid exchanges. Because of the small number of chromosomes these diploid metaphases are ideal for determining the number of sister chromatid exchanges. For example, one chromosome I (Figure 4) shows no exchanges while the other has three. One chromosome II near the center of the group has one exchange and the other chromosome II at the lower right, which is lying under the chromosome I without exchanges, has two exchanges. However, this may not be evident from the photograph. One of the chromosomes III–IV has no exchanges (near the center of the group); two have one exchange and the one in the left center has two exchanges.

Although sister chromatid exchanges are more easily analyzed in diploid cells like the one shown (Figure 4) than in tetraploid cells, the diploid cells do not give all of the information on the frequency and types of chromosome exchanges. The tetraploid cells at the second division after labeling contain all of the chromosomes derived from the original labeled complement and allow a more complete analysis of the origin of the exchanges.

One of the striking features of these labeled tetraploid cells was the frequency with which twin exchanges occurred in homologous chromosomes (Figures 5 and 6). Twin exchanges are defined as sister chromatid exchanges at the same locus in two separate chromosomes at the second c-metaphase. Since the two chromosomes have been separated since the first c-metaphase the twinning must reflect some event that occurred before the separation of the chromosomes at the first division. With this in mind the number of twin and single exchanges were counted. The frequency of each type is given in Table 1. Since the frequency of single exchanges was relatively low the classification of the two types was quite reliable especially in the two largest chromosomes. In the smallest chromosomes, two single exchanges may occasionally occur near enough the same locus in two of the eight chromosomes to be mistaken for a twin. Confidence that exchanges are really at the same loci in two separate chromosomes is gained by observation of instances in which two sets of twin exchanges in one pair of chromosomes match exactly (Figure 8, chromosomes II).

Exchanges were recorded (Table 1) only when all four of the homologous chromosomes in a particular cell could be analyzed. This meant that a considerable number of cells at the proper stage of colchicine treatment, which were sufficiently labeled to produce autoradiographs, could not be used because some

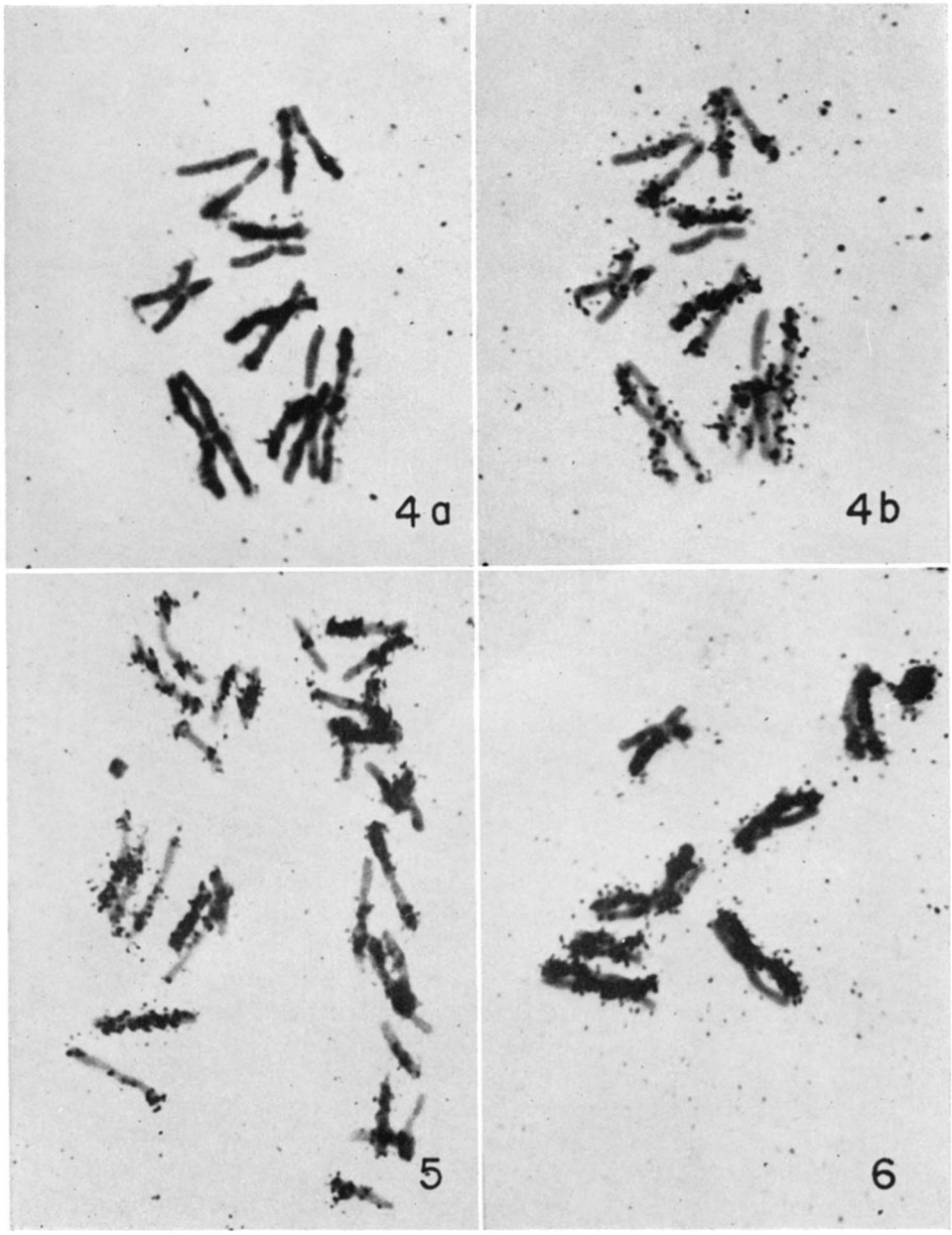

FIGURE 4.—C-metaphase at the second division after labeling (diploid cell). × 1650. 4a—Focal level of chromosomes. 4b.—Focal level of grains.

FIGURE 5a.—C-anaphase at the second division after labeling (tetraploid cell) when the sister chromatids have completely separated. × 1500.

FIGURE 6a.—Part of the complement of a tetraploid c-metaphase at the second division after labeling before chromatids separate. × 1500.

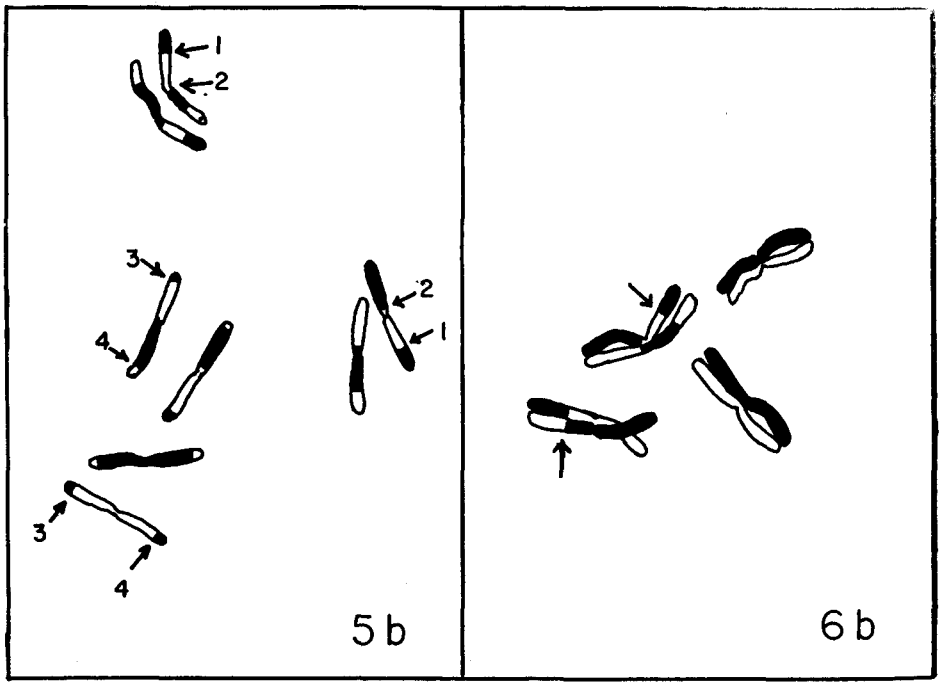

FIGURE 5b.—Drawing of chromosomes I from FIGURE 5a showing exchanges; twins indicated by arrows (black regions represent labeled portions).

FIGURE 6b.—Drawing of chromosome I from FIGURE 6a to show the position of exchanges; twin exchanges indicated by arrows.

TABLE 1

The frequency and types of exchanges observed at the second c-metaphase in root tip cells of Bellevalia

Chromosome	Number of chromosomes examined (chromatid pairs)	Number of twin exchanges	Number of single exchanges	Mean frequency of exchanges per chromosome	
				1st interphase (twins)	2nd interphase (singles)
I	72 (18 cells)	36	15	1.00	0.21
II	52 (13 cells)	17	4	0.66	0.08
III, IV	80 (10 cells)	28	11	0.70	0.14
Totals	204	81	30		

of the four chromosomes were lying under or over some others of the complement. Some cells were analyzed after they were at c-metaphase long enough for sister chromatids to become separated and sometimes displaced from their sisters (Figure 5). By the pattern of exchanges and morphological features of the chromosomes a reconstruction of the original relationships could sometimes be made, especially if part of the sister chromatids were still adjacent to each other. Figure 8 shows the reconstruction of the complement from such a cell.

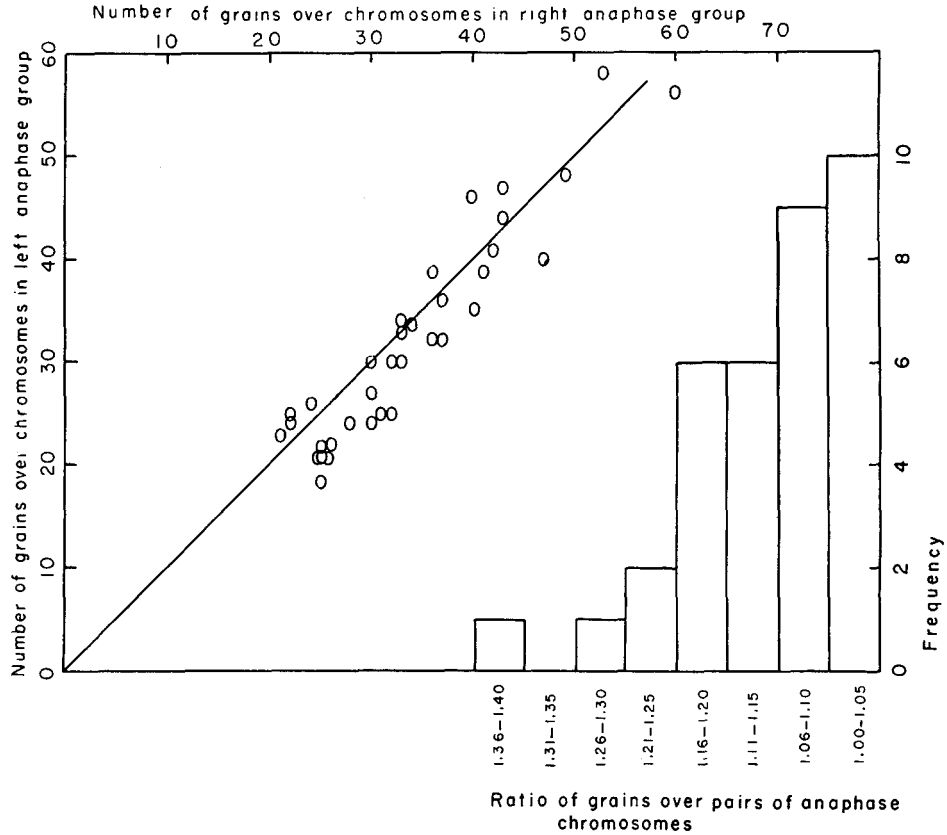

FIGURE 7.—Graph showing the ratio of grains (reflecting radioactivity in the chromosomes) over sister chromosomes in anaphase at the first division after labeling.

Twin exchanges did not occur in the diploid c-metaphases (Figure 4). Their failure to appear in these cells shows that the breaks are not localized by some mechanism which would simulate twin exchanges, and therefore, the observation adds to the confidence of the interpretation of twins. Among 72 chromosomes I examined (18 tetraploid cells), there were 36 twin exchanges (72 chromosomes with exchanges) and 15 single exchanges (Table 1). For chromosome II, 17 twins and four singles were observed among 52 chromosomes (13 tetraploid cells). Altogether among 204 chromosomes examined, there were 81 twins to 30 single exchanges. Interpretation of the frequency of exchanges per chromosome will be deferred until a consideration of the types of exchanges possible is made (see discussion).

Distribution of the isotope at the second division was regularly all or nothing as previously stated and nearly all breaks appeared to be clean cut in so far as resolution will allow determination of this feature. However, a few exceptions noted are worth recording. Among 204 chromosomes analyzed for exchanges at

the second division, two instances of apparently equal distribution of the isotope to both chromatids from the locus of an exchange to the end of the chromosome were noted. One instance of equal distribution in an interstitial segment between two exchanges was seen also. One chromosome in a cell not recorded in Table 1, because not enough of the chromosomes were lying separately, showed equal distribution for the whole length of one chromosome. All others in the complement that could be analyzed showed the typical all or nothing segregation. In all of the instances the regions with equal distribution show the isotope to be about equal in amount in the two chromatids to that in a single chromatid of the remainder of the complement. In spite of the above mentioned discrepancies no instance was found in which opposite segments of both chromatids were unlabeled. This confirms the observation that unlabeled segments do not occur at the first division, for if they did the result at the second division should be that both sister chromatids would be free of label in some segments of the chromosomes.

Although no effort was made to determine quantitatively the dose of the endogenous radiation from the tritium, it was enough to produce aberrations. A fragment may be seen at anaphase of the first division in Figure 3. In addition among about fifty metaphases examined for sister chromatid exchanges, four interchromosomal exchanges were seen. All of these were of the chromosome type, i.e., the rearrangements involved both chromatids in each chromosome. Of course, they could have been derived from the duplication of chromosomes in which chromatid exchanges had occurred at the first division.

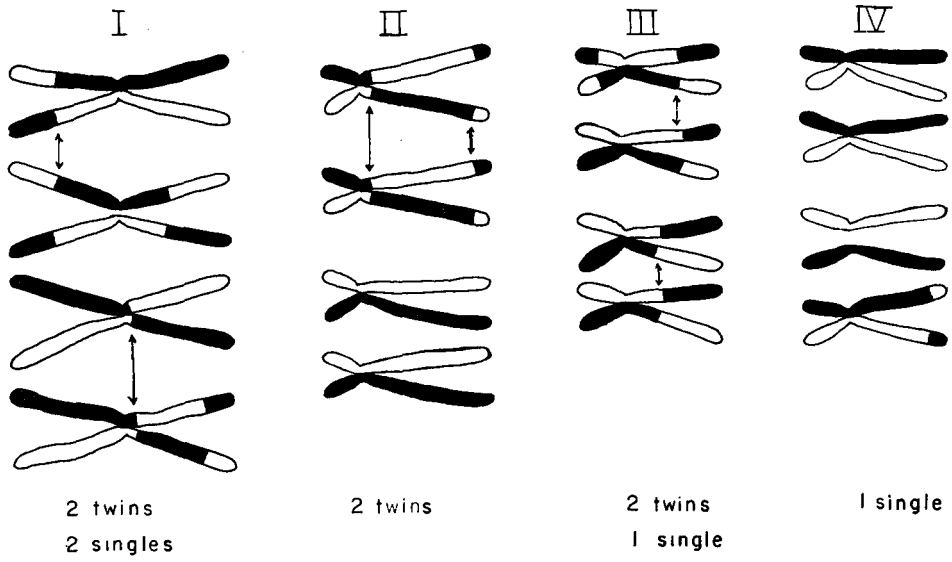

FIGURE 8.—Diagramatic reconstruction of the chromosome complement from a tetraploid c-metaphase at the second division after labeling to show the position of exchanges; twins indicated by arrows.

DISCUSSION

Since each chromatid is a duplex, the possibility of an exchange involving only one strand of each duplex should be considered. If an exchange occurred between a labeled strand of one duplex and an unlabeled strand of the other duplex, anaphase chromosomes at the first anaphase after labeling occurred would contain unlabeled segments (Figure 9a). Since the chromosomes were uniformly labeled

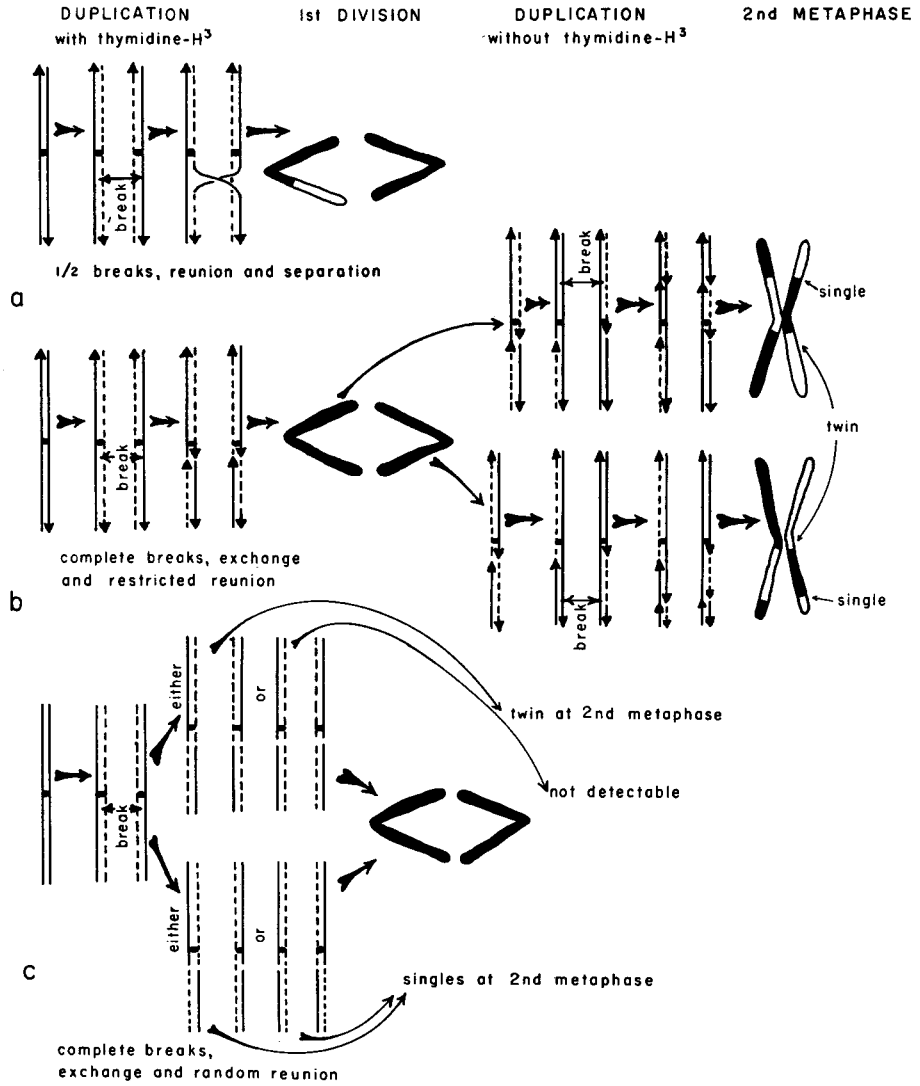

FIGURE 9.—Chart showing the results of chromatid exchanges and the predicted ratio of twin and single exchanges. (a). The predicted results of one-half chromatid exchanges which were not observed. (b). The results of exchanges when reunion is restricted by a difference between the two strands of the chromatids. (c). The results of exchanges when reunion is unrestricted and rejoining of strands occurs at random. Labeled units are shown as dashed lines. The difference between strands is represented as a directional sense and is indicated by arrows.

at this stage, we conclude that this type of exchange did not occur. Instead all exchanges observed resulted from the breakage of whole chromatids, i.e., both strands of each duplex broke at the same or nearly the same locus.

Half-chromatid exchanges have been described a number of times following irradiation of cells since they were first reported by NEBEL (1936) and by SWANSON (1947). They are seen as anaphase bridges without accompanying fragments. Photographs published by CROUSE (1954) and by LACOUR and RUTISHAUSER (1954) are particularly clear. However, aside from these observations, there is no direct experimental evidence concerning the mechanism of the origin and disposition of these bridges. ÖSTERGREN and WAKONIG (1954) obtained negative evidence that the half chromatid exchanges did not occur, i.e., that the "stickiness" observed at anaphase involved only the matrix of the chromosomes. This conclusion was based on the failure of the half exchanges to appear as whole chromatid exchanges at the next division when roots were grown in colchicine to prevent the breakage of the connections at anaphase. Neither their evidence nor the observation of labeled chromosomes reported here demonstrates that the half exchanges do not occur. These experiments only show that they do not persist either after a normal anaphase or after a c-mitosis. They could be first events in whole chromatid exchanges. Their occurrence might produce an unstable condition which either undergoes restitution or results in breakage and exchange of whole chromatids at some time before the second c-metaphase.

The occurrence of the twin exchanges in the tetraploid c-metaphases and their absence in the diploid c-metaphases indicate that they are due to events occurring before the separation of the sister chromatids at the first division after labeling. This leads us to a consideration of the types of exchanges that can occur between duplexes (chromatids) each of which has one labeled strand and one unlabeled strand. We will discuss two possibilities: (a) that reunion is restricted by a difference in the two strands of the duplex, and (b) that reunion is unrestricted (Figure 9).

If the two strands of the duplex are different in some way, for example in directional sense analogous to the two strands of the WATSON and CRICK (1953) model of DNA, the types of reunion would be restricted (Figure 9b). If we suppose the two strands have opposite sense, and that only strands of like sense can rejoin when exchange occurs, a labeled and an unlabeled strand will regularly rejoin in each chromatid. At the first division both daughter chromosomes will appear labeled throughout their length. After another duplication in the absence of labeled precursor the exchange will be revealed. Each two c-metaphase chromosomes, descended from a chromosome with the type of exchange described above, will have sister chromatid exchanges at the same locus, i.e., twin exchanges will appear at the second division. Each of these second division chromosomes may also contain single exchanges which can occur after the second duplication. Since at this stage only one of the chromatids has a labeled strand at any level, each exchange will become visible at the division immediately following, i.e., the second c-metaphase. If we assume that the same probability for breakage and

exchange exists at both the first and second divisions, we predict a ratio of one twin to two singles according to the following reasoning. Let the probability of a break in both chromatids of a chromosome during the first interphase when labeling occurs be p. For the second interphase let the frequency be q and assume that the same chance for exchange or restitution of the breaks occurs in both interphases. Further, assume either (1) the rejoins are restricted by a difference in the two strands and only like strands can rejoin, or (2) that the rejoining is not restricted and the four ways to rejoin are equally possible (Figure 9c). When p and q are equal the ratio expected for twins to singles with restricted rejoining is 1:2, and for unrestricted rejoining the ratio is 1:10. The latter ratio is the minimum figure for if unrestricted rejoining could occur a break in a single chromatid with rotation of 180° and rejoining at any time before the second interphase duplication would produce single exchanges. Their frequency cannot be predicted, but if they could occur the ratio of twins to singles would be a fraction considerably smaller than 1:10. When q equals zero, i.e., no exchanges occur in the second interphase, the ratio would be 1:0 for restricted rejoining and 1:2 for unrestricted rejoining. An examination of the data in Table 1 shows that both twins and single exchanges were observed at the second c-metaphase in tetraploid cells, i.e., when all of the descendants of the original complement of labeled chromosomes could be examined. However, the number of singles is considerably less than predicted on either the restricted or unrestricted rejoining hypothesis when p equals q, i.e., when the frequency of breakage and exchange is equal in the two interphases. However, the hypothesis of unrestricted rejoining can be eliminated because the frequency of twins is several times higher than would be predicted even when no exchanges occurred in the second interphase. Unrestricted rejoining would produce a ratio of one twin to two singles while the observed ratio was 81 to 30. The conclusion must be that rejoining is restricted and the frequency of exchanges in the second interphase is lower than in the first interphase.

With the knowledge that the rejoining is restricted and that all exchanges in the first interphase produce twins, a calculation of the frequency of exchanges at the two divisions can be made (Table 1). The frequency of sister chromatid exchanges for chromosome I is 1.0 in the first cycle and 0.21 in the second cycle. For the smaller chromosomes the frequency is lower, 0.66–.70 exchange per chromosome in the first cycle. The lengths of the chromosomes are in the ratio 100:75:65 for chromosomes I, II, and III–IV, respectively. Therefore, the frequency of exchanges is very nearly proportional to length.

The lower frequency of exchanges in the second interphase can be correlated with two differences in the two cycles. The amount of endogenous radiation is at least reduced by one half at the second interphase. Perhaps the difference is even greater if the thymidine derivatives enter the cell but are not utilized. By the second interphase they might be transported to other parts of the plant or lost into the culture solution. The other difference is that the second interphase occurs in the presence of colchicine while most of the first interphase is completed before the roots are placed in colchicine. BRUMFIELD (1943) reported that colchi-

cine reduced by two thirds the chromatid aberrations induced by X-rays. It appears possible that colchicine might also reduce any naturally occurring sister chromatid exchanges as well as radiation induced ones. Although it is not possible to predict what fraction of the exchanges, if any, occur naturally, the conclusion with respect to restricted reunion stands. Even if all of the exchanges were occurring in the first interphase the number of singles is far less than would be predicted on the basis of unrestricted reunion.

Therefore the interesting and significant conclusions are (1) that all breaks are four strand breaks and reunions and (2) that the two strands of the chromosomal duplex are not alike. The doubleness is almost certainly a fundamental property of all chromosomes. No matter how many DNA and protein chains may be present in a chromosome, these must be organized into two units that are opposite in some sense. This oppositeness could be either a mirror image difference between the two strands or a difference in directional sense with or without complementarity. However, since the two units of the duplex fit together so intimately that the new and old ones rarely if ever separate at the division immediately following duplication, they are likely to be complementary in the chemical sense.

The two polynucleotide strands of the WATSON-CRICK model of DNA are different in directional sense and therefore are analogous to the chromosome in this respect. A chromosome thus might be a single double helix. However, the possibility that the chromosomal duplex is composed of *two* WATSON-CRICK double helices is certainly eliminated, for these would be identical.

To obviate the problems of coiling such a long strand (over one meter in large chromosomes) of DNA into a chromosome and of unwinding the stands during duplication, TAYLOR (1957) has proposed that the chromosome is an array of DNA double helices attached to a central core. The core is visualized as a double ribbon with DNA double helices attached along the edges so that each helix has one polynucleotide chain attached to one ribbon and the other chain of the same helix attached to the other ribbon, perhaps by a terminal phosphate group. Each ribbon with its attached DNA chains, possibly on both edges to make it symmetrical, would comprise one unit of the duplex. The duplex chromosome would separate into its components during duplication. Another ribbon would be built along each separating ribbon and the DNA double helices would begin to pull apart, with the separation from each other starting at their points of attachment to the ribbons. With the unattached end free to rotate and with rotation of strands possible at single bonds along the polynucleotide chains in the separating regions, replication could proceed according to the WATSON and CRICK scheme. All new polynucleotide chains would be attached to the new ribbons and would segregate as units in future duplications of the chromosomes, while the sister chromatid exchanges would represent breaks and exchanges along the axis.

At what stage in the cell cycle do the sister chromatid exchanges occur? Are they formed as a part of the duplication process and are they mechanically comparable to crossovers? The analysis indicates that the exchanges occur either during duplication or between the time of duplication and separation of daughter

chromosomes. Since the two strands of the chromosome are different, no strand exchanges can occur between the time of separation and the time of the next duplication. Duplication then produces strands that can exchange with the original ones, although the two new strands cannot exchange with each other; they are unlike. As mentioned earlier exchanges between two of the four strands might occur, but this requires a breakage of at least one of the original strands. Such half exchanges might be more likely during duplication, but nothing observed so far would limit exchanges to the period of duplication. Half exchanges if they occur at all do not persist until the second c-metaphase. Mechanically the exchanges may be comparable to crossing over, but the evidence is not compelling. Any study of crossing over must, of course, take into account the difference in the two strands of the chromosome.

SUMMARY

By following the distribution of tritium-labeled DNA in chromosomes of Bellevalia, they are shown to be two-stranded before duplication. Both chromatids of each chromosome are labeled at the first metaphase or anaphase after incorporation of tritium-labeled thymidine. After another duplication in the absence of labeled precursors each labeled chromatid (chromosome now) yields one labeled daughter and one free of label at any level along the length. Sister chromatid exchanges were frequent, but half-chromatid exchanges, i.e., exchanges between one strand in each of two chromatids, were not observed. Sister chromatid exchanges were frequently observed to occur as pairs or twins, i.e., exchanges occur at the same locus in two of the four homologous chromosomes in the second c-metaphase after labeling. Among 204 second metaphase chromosomes examined there were 81 twin exchanges and 30 single exchanges. This frequency can be explained only on the hypothesis that the two strands of the chromosome are unlike, i.e., are not free to reunite at random. Therefore, the chromosome has two features in common with the WATSON-CRICK model of DNA. It has two strands and the strands are different in some structural feature that restricts reunion to like strands when chromatid exchanges occur.

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